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**ABSTRACT**

*Leptomyxa valladarensis* was isolated from soil in a pine forest on the southern flank of Mt Teide in Tenerife, Spain. It feeds on bacteria and on a range of other amoebae, and it was possible to establish bi-axenic cultures with *L. valladarensis* and *Acanthamoeba*. It is easily propagated on a *E. coli* also. 18S rDNA gene sequence analysis suggests that it is most closely related to *Leptomyxa variabilis*, however this amoeba differs in important detail. *L. valladarensis* is primarily mononucleate whereas *L. variabilis* is multinucleate. *L. valladarensis* is a larger amoeba and although the cysts are similar in size, there is no sign of the pore-like structures described in *L. variabilis* cysts. *L. valladarensis* can adopt a rapid monopodal and tubular morphology similar to that described for *L. neglecta* and *Rhizamoeba matisi*, and is never reticulated as larger *L. variabilis* individuals tend to be. The mean generation time was found to be 18 hours, in line with amoebae of this size. Like other members of the genus, *L. valladarensis* is reported to harbour intracellular, presumably endosymbiotic bacteria, and a *Delftia* sp has been identified by 16S PCR a bacterium which is also known to grow within *Acanthamoeba*. The availability of this easily cultured species will help to characterize of this little studied genus and family and their relationship with bacteria, both prey and symbionts.

**Keywords**: Amoebozoa; Leptomyxida; Systematics; Phylogeny.

## 1. Introduction

Amoeboid organisms belonging to the order Leptomyxida have been isolated from a variety of environments including saltwater (Page, 1983; Kühn, 1996), freshwater (Smirnov, 2009), temperate soils (Brown & Smirnov, 2004), soils above the permafrost (Shmakova et al., 2013), and soils from deserts (Bamforth, 2008). Order Leptomyxida contains an unusual assortment of medium to very large amoebae some attaining a length of 3mm in some cases, making them amongst the largest amoeboid organisms (Singh, 1948). This group display a variety of different morphologies, not only between species but individuals within the same species are

often found to adopt very different morphologies and to be able to switch rapidly between them. Some members produce fan-shaped flattened trophozoites (similar to the Vannellae), some that produce adhesive root-like ramose processes, and others that produce simple tubular, limax forms which advance rapidly, led by a hemispherical hyaline cap. As a result of this heterogeneity in form and habit, many genera and species have been described and the historic state of the *Leptomyxida* was one of disarray (Smirnov et al., 2008; Smirnov et al., 2009).
A recent comprehensive phylogenetic and morphological analysis of the *Leptomyxida* has brought clarity (Smirnov et al., 2017). The order now been reorganised into three genera. Genus *Flabellula* (uniting the genus *Paraflabellula*, some members of genus *Rhizamoeba* and the pre-existing genus *Flabellula*), genus *Rhizamoeba* (presently consisting of one named species, *R. saxonica*, CCAP1570/2) and genus *Leptomyxa*. A small number of the genus *Leptomyxa* have been described (Table 2) and only two are currently available in the main culture collections. The strain described here is easy to culture and maintain, and its availability will hopefully help characterize this interesting group.
Free living amoebae (FLA) are known to produce infection in humans (Schuster and Visvesvara, 2004; Visvesvara et al., 2007), one of these, *Saccamoeba* is within the Tubulinea (Gelman et al., 2001; Walochnik et al., 2010. The heterolobosean, *Naegleria fowleri* causes a usually fatal meningitis (Carter 1970), and while the T4 genotype of *Acanthamoeba* is the most abundantly encountered type it is also known to be disproportionately associated with human infection (Maciver et al., 2013), the most common of which is *Acanthamoeba* keratitis (Lorenzo-Morales et al., 2015). *Balamuthia mandrillaris* is a third major FLA which causes human fatalities (Maciver 2007). This amoeba was first described as being a Leptomyxid amoebae because of its superficial resemblance to this group (Visvesvara et al., 1990), but it is presently clear that genus *Leptomyxa* and the groups immediately related to it, do not contain pathogens. Even if FLA species are not directly pathogenic many harbour intracellular bacteria that may be human pathogens (Mohamed et al., 2016). It is therefore important to understand FLA diversity and their interaction with bacteria (Walochnik et al., 1999).

## 2. Methods and Materials

### 2.1 *Cell culture*

*Acanthamoeba* (Neff strain, ATTC30010, CCAP1501/1B) was cultured axenically in 75 ml flasks. *Vermamoeba vermiformis*, *Vannella pentlandii* (CCAP 1589/23) and an unidentified vahlkampfiid amoebae were cultured on YME plates (2% agar, 0.01% yeast extract, 0.025% malt extract) with *E. coli* as previously described (Maciver et al., 2018). The yeasts *Pichia pastoris* and *Rhodotorula mucilaginosa* were cultured on 1.5% agar plates with 0.01 % malt extract and 0.01% yeast extract. *E. coli* (BL21 de3) was cultured on LB plates or grown in liquid LB media.

### 2.2 *Isolation of amoebae*

A small sample of dry soil was taken from a dry gully in a large forested area consisting predominantly of the Canary Island Pine (*Pinus canariensis*) from a site known, (not very helpfully) as “Las Lajas” meaning “the Rocks” at lat.28.1886º long. -16.6644 º and at an altitude of 2076 m on the southern flank of Mount Teide, Tenerife, Canary Islands, Spain. Amoebae were isolated by plating soil in Neff’s saline plates overnight and then inverting small strips onto fresh non-nutrient agar plates layered with *E. coli*. After several days *Acanthamoeba* was found to grow out, and a much larger amoeba (strain LLT) was subsequently observed to grow out over the *Acanthamoeba* dominated regions of the plate, until very few *Acanthamoeba*

could be found. *Acanthamoeba* were easily identified on the plates by the characteristic appearance of the cysts. LLT was initially cloned by excising small regions off agar plates and placed in micro wells containing trophozoites of *Acanthamoeba* (Neff strain). LLT was found to grow on a bacterium from the original source and was routinely cultured with this bacterium or *E. coli* (BL21 de3) on YME plates (see above).
For long term storage amoebae and cysts were washed in Neff's saline and then taken up in 12% DMSO in Neff's incubated at room temperature, then overnight at -20°C then finally placed at -80°C. Cultures were revived by thawing out to room temperature, washing with Neff's saline by low speed centrifugation, and either fed with *Acanthamoeba* in Neff's saline to a culture flask, or placed on *E. coli* spread YME plates. Additionally, LLT were stored at room temperature in clay pellets as previously described (Lorenzo-Morales & Maciver, 2006). Light micrographs and measurements were performed on a Leica inverted microscope with Hoffman modulation contrast microscope and a laptop controlled Canon EOS digital camera. Additional micrographs were taken using a phase contrast microscope. Videos were recorded using "EOS camera movie record 0.3" (eos-movrec.sf.net). For the enumeration of nuclei, amoebae were fixed with Nissenbaum's fixative (Nissenbaum, 2001) and stained with Kernechtrot nuclear fast red (Fluka).

### 2.3 *Genomic DNA purification and PCR*

Genomic DNA was isolated and purified as previously described (Lorenzo-Morales et al., 2005; Maciver et al., 2018). The eukaryotic 18S rDNA gene was amplified using several primer pairs as previously described (Corsaro and Venditti, 2010). CAT1 (5′-CAT GCA TGT CTA AGT ATA AGC-3′) with GSPr (5′-TTC AC <G/A> GTA AAC <G/A> ATC TGG GC-3′), or 1137R (5′-GTG CCC TTC CGT TCA AT-3′), and 892cF (5′-GTC AGA GGT GAA ATT CTT GG-3′) with Br (5′-GAT CCT TCT GCA GGT TCA C-3′). Bacterial 16S rDNA genes were amplified using universal prokaryote primers 16S Fwd 5′-GTT TGA T<C/T> <C/A> TGG CTC AG-3′ and 16SRev 5′-CA<T/G> AAA GGA GGT GAT CC-3′ (Horn et al., 2002). PCR conditions were 15 min at 94°C, followed by 30 cycles of 94°C for 30 s, 55°C 30 s, 72°C 2 min, with final extension of 72°C for 5 min, except for the 16S PCR where the annealing temperature was increased to 58°C. The resulting PCR products were sequenced by Edinburgh Genomics with the same primers as were used to amplify them plus internal primers where necessary.

### 2.4 *Phylogenetic analysis*

18S rDNA Sequences were obtained for genus *Leptomyxa* and relatives from Genbank and compiled together with the sequences obtained in this study using "Seaview", version 4 (Gouy et al., 2010). The program "BioEdit" (Hall, 1999) was used to edit sequences where needed and to determine levels of homology between sequences. Maximum likelihood phylogenetic trees were obtained using PhyML software (Guindon and Gascuel 2003) using the GTR model implemented with Seaview version 4. The non-parametric analysis was performed with 100 bootstrap pseudo-replicates, using sequences from other Leptomyxids as the outgroup. Sequences obtained in this study were submitted to GenBank and are available under accession number KX792145 for the 18S rDNA gene, and MF441175 for the 16S rDNA endosymbiont sequence.

## 3. Results

Strain LLT was isolated by the usual "walk out" method in which amoeboid organisms crawl out from the sample on a lawn of (usually) *E. coli* (Neff, 1958). This encourages the isolation of small fast growing amoebae (most often *Acanthamoeba* and *Naegleria*). We noticed that a small number of large amoebae were feeding on the *Acanthamoeba* that preceded them and were able to isolate this amoeba by cloning. LLT was found to be reluctant to grow on agar surfaces presumably due to the very thin layer of water on the surface of these plates, however it was successfully isolated from such plates when *Acanthamoeba* sp was present. Initially it could not grow on *E. coli* when placed on *E. coli* spread non-nutrient agar plates, nor did it grow when presented with *E. coli* on flat bottomed flask in Neff's saline. However, after a year of growth on accompanying bacteria, LLT was found to be able to grow well on *E. coli* alone, with a mean generation time of 18 hours. Although LLT was seen to phagocytose yeast when they were presented, neither *Pichia pastoris* nor *Rhodotorula mucilaginosa* alone could support the survival of this amoeba. It did however grow rapidly when placed with *Acanthamoeba* (Neff strain) in Neff's saline. A small vahlkampfiid amoebae, *Vermamoeba vermiformis* and *Vannella* were seen to be phagocytosed by LLT but as they were also in the presence of *E. coli* it was not clear if these amoebae alone could support the growth of LLT. LLT was also observed to phagocytose the hyphae of an unidentified fungus, however it was not clear if fungi alone could support the growth of LLT as this fungus overgrew cultures causing LLT to encyst.

Phylogenetic analysis of the 18S rDNA gene revealed that LLT groups with genus *Leptomyxa* (Figure 4) and that it is most closely related to *L. variabilis*. The 18S rDNA sequence and the unique morphological features make it clear that LLT is a new species of *Leptomyxa* and we have named it *L. valladarensis*.

Monoaxenic cultures were established with axenically grown *Acanthamoeba*, by overnight treatment of LLT trophozoites and cysts with gentamycin (10µg/ml) and ampicillin (100 µg/ml) in Neff's saline. These cultures were maintained at room temperature and supplemented with fresh *Acanthamoeba* when required. The LLT cysts are variable in size (Table 1, Figure 2d) with a mean diameter of 23 µm. Most cysts contained a single nucleus, a few had double nuclei and fewer still contained three (Figure 2d; Table 1). Perhaps as expected those with supernumerary nuclei tended to be larger, as is the case for other amoebae (De Jonckheere, 2006), yet not it seems for *Leptomyxa reticulata* (Cann, 1984). The trophozoites were very large as is typical for the genus (Table 2) and when locomotion was organized and rapid, short uroidal filaments were often visible (Figure 1) and a tube shaped monopodal morphology adopted (Figure 2b).

Overall the locomotory habit was similar to that described for other members of this order. The amoeba was variable in locomotory habit, changing rapidly for example from a fast moving cylindrical (limax) form with a single pseudopodium (Figure 2b) to a flatter broader shaped form with a leading front which expanded on a number of different fronts often with changing dominance (Figure 1 & 2a). The pseudopod into which the nucleus entered became dominant (Videos 2 and 3, see supplemental data) in a manner similar to that reported for *Balamuthia mandrillaris* (Dunnebacke, 2009). Nuclei drawn into these pseudopods were seen to deform as they did so, taking on temporary oval or cylindrical shapes. Simple monopodal forms were observed to stop and to travel in the opposite direction by the creation of a pseudopod from the former uroid in a manner similar as that described for other amoebae such as V*ahlkampfia inornata* (Page, 1967).

Forward advance often occurs by protrusion of broad, hyaline often hemispherical bulges which erupt forward in a manner which is normally considered to be a hallmark of the heterolobosean amoebae. This similarity between some of the family Leptomyxidae and the class heterolobosea has of course been noted by others (Page, 1988) and it is suggested that

some current amoebae presently labelled as *Rhizamoeba*, (such as *R. schnepfii*) may actually be heteroloboseans (Smirnov, 2009). Another shared feature is that the liquid hyaline ectoplasm tends to run down the side of the gelled endoplasmic cortex under the plasma membrane and then "setting", or becoming gelated. This has the appearance of candle wax spilling down the candle shaft and suddenly solidifying as it cools.
There was no particular floating form as described for *R. matisi* (Smirnov et al., 2017), instead amoebae kept in suspension for 2 hours adopted a similar shape as they did will crawling but with blunt projections branching off the main body at all angles and in constant motion (Figure 3). These quickly resumed locomotion as they settled on the vessel floor.

## 4. Discussion

In common with many groups of protozoans a congruence is sought between morphological features and specific sequence data in the genus *Leptomyxa* (Dykova et al., 2008; Smirnov et al., 2017). The genus *Leptomyxa* as it presently stands is composed of six named species and these are all terrestrial (soil/freshwater) members. The existence of further members is indicated by sequences from environmental samples (Smirnov et al., 2017). Despite the observed similarity in locomotory morphology between *Rhizamoeba* / *Leptomyxa* and the Heteroloboseans, this genus is classified (Amaral Zettler et al., 2000; Tekle et al., 2008) as belonging to the family Leptomyxidae part of the Tubulinea clade within the super group 'Amoebozoa' together with well-known genera such as *Amoeba* and *Acanthamoeba*. The fact that the core *Rhizamoeba* strains group so closely with *Leptomyxa reticulata* is a puzzle since these organisms appear to be so very different at the light microscope level and in ultrastructure (Cann, 1984). As its name suggests *L. reticulata* is a reticulate amoeba often with hundreds of nuclei scattered throughout its many anastomosing pseudopodia (Cann, 1984; Page 1988). Doubt has been expressed (Smirnov et al., 2009) if strain ATCC 50242 (Amaral Zettler et al., 2000) was correctly assigned to the species *Leptomyxa reticulata* since it is so dissimilar to other Leptomyxids. However, more recently, other species which have anastomosing reticulopodia have been isolated and 18S rDNA gene sequences analysis agrees that *L. reticulata* groups together with *L. arborea* (Smirnov et al., 2017).
An analysis of the available 18S sequences revealed that the *L. valladarensis* 18S gene is most similar to *L. variabilis* (Smirnov et al., 2017) which differs in only 2 nucleotides from the "*Ripidomyxa*" 18S gene. However, the situation is complex as the sequence deposited in Genbank (AY549563, AY549564) is not actually the isolate named as "*Ripidomyxa australiensis"* (Chakaroborty & Pussard, 1985), although they seem to share very similar morphology (Hewett, 2006). Additionally, the *Rhizamoeba australiensis* available as CCAP 1570/4 is also a different isolate to either Australian isolate and in fact originated in roof gutter sediment in Melsbach, Germany. *Rhizamoeba australiensis* CCAP 1570/4 is now reclassified as *Leptomyxa australiensis* (Smirnov et al., 2017).
The generation time of 18 hours compares with the 46 to 83-hour generation of *Amoeba proteus* (Rogerson, 1980). 4 hours for *Dictyostelium* (Fey et al., 2007) and for *Naegleria* (Chang 1958), 6 hours for *Acanthamoeba* (Jensen 1970) *Mayorella* (90-160µm in length) with generation times 41.6h (Laybourn-Parry et al., 1987). We found that there was a simple relationship between the length of an amoebal species and its generation time (Figure 5). Such a relationship is to be expected as larger amoeba need to attain more mass than smaller amoebae in order to divide, and a similar relationship (generation time = 0.311 $\text{Log}_{10}$ cell volume ($\mu m^3$) + 0.0445) has been reported (Baldock et al., 1980). The nuclei of *L. valladarensis* are large but usually single. Most other members of the genus have more than one nucleus presumably to produce the RNA required for cell growth and maintenance. The other function of the nucleus

is to store the genome but there is no *a priori* reason the genome of *L. valladarensis* should be larger than a smaller amoeba such as *Acanthamoeba* which has a similarly unambitious life style. The (usually) single nucleus of *Amoeba dubia* has around 20,000 genome copies (Friz, 1968), and so is highly polyploid. Most amoebae reproduce asexually (Lahr et al., 2011), but asexual reproduction risks the accumulation of mutations by a process named "Muller's ratchet" (Muller, 1964). It has been argued that amoebae are polyploid and that together with recombination this prevents the operation of Muller's ratchet (Maciver, 2016). There is no evidence for sexual reproduction in *L. valladarensis* (or any species in this genus) and so it is likely that the large nucleus of this amoeba is polyploid to permit persistent asexual reproduction.

Leptomyxid amoebae have proven difficult to isolate and to maintain in culture (Smirnov et al., 2009). *L. valladarensis* thrives on *Acanthamoeba* as a food source and this di-axenic system is convenient to propagate this amoeba. However, it also grows very well on *E. coli*. The feeding habits of *L. valladarensis* are typical of amoebae of this size and it is not surprising that *Acanthamoeba*, *Vermamoeba* and other small amoebae serve as food. *Rhizamoeba* are reported to engulf protists (Rodriguez Zaragoza et al., 2005) and *Rhizamoeba matisi* is also known to consume *Vermamoeba vermiformis* (Smirnov et al., 2017). The original "*Ripidomyxa australiensis"* strain was also able to grow in the presence of a single bacterium (Chakaroborty & Pussard, 1985), but this strain has been lost.

The observation that all cultures of *L. valladarensis* produce a geosmin-like smell independent of the food organisms present is similar to the case of the "*Ripidomyxa*" isolate (Robinson et al., 1995). The origin of geosmin was attributed to a symbiotic bacterium (Robinson et al., 1995; Hewett, 2006). 16S sequesnces from presumed endosymbiont bacteria from this strain (AY549554 and AY549555) had no matches in 2006 (Hewett, 2006), nor do they to date (July, 2017). PCR using universal prokaryote16S primers revealed that *Delftia* or a related bacterium was present within *Leptomyxa valladarensis*. *Delftia* (a.k.a. *Comamonas*) is also known to infect *Acanthamoeba* (Walochnik et al., 1999). The production of geosmin by amoebae in culture has been reported in *Vannella* (Hayes et al., 1991). The significance of the production of geosmin is uncertain but volatile organic compounds affect amoeba prey selection (Schulz-Bohm et al, 2017), and it is possible that it has an anti-predator role (Höckelmann et al., 2009).

*Diagnosis.*

*Leptomyxa valladarensis* n. sp. The amoeba adopts two main locomotory morphologies. It is transiently limax with a long, simple, tubular shape where the length is 70 - 180 µm; breadth 20 – 60 µm only occasionally does this form have an adhesive uroidal filaments or villous bulbous uroid. In limax locomotion the frontal hyaline cap occupies up to 1/5 of the total length of locomotive cell. The more usual locomotory habit is a flattened multi pseudopodal form and this form may have adhesive uroidal filaments and/or villous bulbous uroid but these are not as prominent a feature compared to reports for other leptomyxids. The amoeba is predominantly uninucleate but larger individuals may (rarely) have as many as 4 nuclei in old cultures. Nuclei are spherical 9 µm in diameter (range 6 to 11 µm). but can become oval shaped as they a compressed within a thinner part of the cell. The cyst (19 – 28 µm in diameter) is again usually uninucleate and is apparently single walled by light microscopy. The cysts can be stored cryogenically in the usual manner. There are no pores visible in the wall by light microscopy.

*The type location.*

A dry gully in a large forested area consisting of the Canary Island Pine (*Pinus canariensis*) from a site known as "Las Lajas" (the rocks) on the southern flank of Mount Teide, Tenerife, Canary Islands, Spain. Site coordinates are lat.28.1886º long. -16.6644º altitude 2076 m above

mean sea level. A fixed slide has been deposited with the Museum of Natural History, London (registration number NMHUK 2016.9.16.1).

*Differential diagnosis.*
Resembles *L. variabilis* in size and morphology, but *L. variabilis* is multicellular whereas *L. valladarensis* is predominantly mononucleate. It also resembles *L. australiensis* in size and organization of the locomotive form, and in the general size of the cyst (*L. valladarensis* being slightly smaller). In common with most Leptomyxid amoebae (Smirnov et al., 2017), a firm identification of *L. valladarensis* is only possible by sequencing the 18S gene.

*Type material.*
The live strain is held at the Culture Collection of Algae and Protozoa at Oban, Argyle, Scotland, with the accession code CCAP 1546/4. 18S rDNA gene sequences obtained from the type strain were deposited with GenBank accession number KX792145.

*Etymology.*
The species name *valladarensis* is to honour Professor Basilio Valladares on the occasion of his retirement after many years of dedicated service to the advancement of parasitology.

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**Appendix A. Supplementary data**

Supplementary material related to this article can be found in the online version

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| | Single nucleus | Double nuclei | Triple nuclei |
|---|---|---|---|
| Diameter (µm) | 22.42 | 28.91 | 35.62 |
| Frequency (%) | 77% | 16.4% | 6.6% |

**Table 1**

| ***Leptomyxa* species** | **Length (µm)** | **Uni or Multi nucleus** | **Cyst features & diameter (µm)** | **Food** | **Known environment** | **Reference** |
|---|---|---|---|---|---|---|
| *L. neglecta* | 70 – 140 | Multinucleate 1 - 5 | None described | Unknown | Freshwater lake | Smirnov, 2009 |
| *L. australiensis* | 50 -180 | Predominantly single | Single wall, no pores 15 - 40 | Fungi & bacteria | Soil | Chakraborty & Pussard, 1985; Smirnov *et al*, 2017 |
| *L. reticulata* | 500 - 3000 | Multinucleate | Double wall 35 - 60 | Non-pigmented bacteria | Freshwater river | Goodey, 1914; Cann, 1984; Page 1988; Singh, 1948 |
| *L. arborea* | 50 - >1000 | Multinucleate | Double wall multinucleate | Fungi yeast bacteria | Soil | Smirnov *et al*, 2017 |
| *L. variabilis* | 30 - 150 | Multinucleate 1 - 20 | Single wall with pores 15 -25 | Unknown | Soil | Smirnov *et al*, 2017 |
| *L. valladarensis* | 70 - 180 | Predominantly single | Single wall, no pores 19 - 28 | amoebae *E. coli* | Soil | This study |

**Table 2.** Currently recognised members of the genus *Leptomyxa* (Smirnov *et al*, 2017; this study).

**Figure Legends**

Figure 1
Amoebae were fixed with Nissenbaum's fixative and the nuclei (n) were stained with Kernechtrot nuclear fast red. The uroidal filaments (uf) are also seen here.

Figure 2
a) Trophozoites in multi-pseudopod locomotion b) Trophozoite in limax locomotory form with the contractile vacuole (cv) toward the rear. d) Bipolar form with two leading pseudopods. c) Three cysts with one nucleus (left most cyst), two nuclei (middle) and three nuclei (right most cyst).

Figure 3
Floating forms. 10 seconds between the three images showing the dynamic nature of the blunt pseudopods.

Figure 4
Phylogenetic analysis 18S rDNA. PhyML ln(L)=-3625.7 1855 sites GTR 100 replication. 4 rate classes. Tree rooted using a number of related *Flabelulla* and *Rhizamoeba* as the outgroup.

Figure 5
a). The growth of *L. valladarensis* on *E. coli*. b). The mean generation time (GMT) of *L. valladarensis* compared to other amoebae (see main text) as a function of their length. A very simple relationship: GMT (hours) = Length (μm) / 10, is apparent.